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Promoter-Specific *trans* Activation and Repression by Human Cytomegalovirus Immediate-Early Proteins Involves Common and Unique Protein Domains†

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***trans* activation of promoters by viral regulatory proteins provides a useful tool to study coordinate control of gene expression. Immediate-early (IE) regions 1 and 2 of human cytomegalovirus (CMV) code for a series of proteins that originate from differentially spliced mRNAs. These IE proteins are proposed to regulate the temporal expression of the viral genome. To examine the structure and function of the IE proteins, we used linker insertion mutagenesis of the IE gene region as well as cDNA expression vector cloning of the abundant IE mRNAs. We showed that IE1 and IE2 proteins of CMV exhibit promoter-specific differences in their modes of action by either *trans* activating early and IE promoters or repressing the major IE promoter (MIEP). Transient cotransfection experiments with permissive human cells revealed a synergistic interaction between the 72- and the 86-kilodalton (kDa) IE proteins in *trans* activating an early promoter. In addition, transfection studies revealed that the 72-kDa protein was capable of *trans* activating the MIEP. In contrast, the 86-kDa protein specifically repressed the MIEP and this repression was suppressed by the 72-kDa protein. Furthermore, observations based on the primary sequence structure revealed a modular arrangement of putative regulatory motifs that could either potentiate or repress gene expression. These modular domains are either shared or unique among the IE proteins. From these data, we propose a model for IE protein function in the coordinate control of CMV gene expression.**

Infection of permissive human cells with human cytomegalovirus (CMV) leads to an ordered sequential expression of viral genes. The first genes expressed after infection are the immediate-early (IE) genes, which require no prior viral protein synthesis. The expression of IE proteins is required for the initiation of early gene expression since infection in the presence of protein synthesis inhibitors prevents subsequent viral gene expression (8, 26, 44, 45). Studies to date have demonstrated that there are predominantly three distinct segments of the CMV genome expressed at IE times (18, 21, 35, 37, 38, 40, 46). Originating from one of these segments are two regions of abundant IE gene expression designated IE region 1 (IE1) (37, 40) and IE region 2 (IE2) (38, 40). These genes are transcribed from a single promoter regulatory region, referred to as the major IE promoter (MIEP), located upstream of IE1 (3, 38, 42). These two regions code for a family of mRNAs that differ due to splicing and which code for a series of unique but related proteins (35, 38). IE1 codes for the major IE protein which originates from a 1.95-kilobase mRNA (37). IE2 codes for several proteins, some of which share sequences with IE1 (35, 38). The predominant IE proteins from region 2 are the 86- and 55-kilodalton (kDa) proteins. The 55-kDa protein is expressed only under IE conditions, while the 86-kDa protein persists throughout infection. At late times, a 40-kDa protein is expressed from region 2 (35).

Numerous studies have shown that IE proteins function in *trans* activation of promoters (7, 11, 16, 31, 41). However, all these studies dealt either with nonspecific heterologous viral or cellular promoters or with transfection of cells nonpermissive for CMV. In contrast, Staprans et al. (34) demonstrated that a plasmid containing IE1 and IE2 could activate a CMV early promoter in human fibroblast cells. Also, a recent study from our laboratory demonstrated that both IE1 and IE2 were necessary for activation of the CMV pp65 promoter (9). However, the role of specific IE1 and IE2 proteins in activating viral gene expression was not addressed in these studies.

To better understand the regulatory role of the IE gene products during infection, we investigated the ability of IE1 and IE2 insertion mutants and IE cDNA expression vectors to activate CMV IE and early promoters in permissive cells. Examination of the regulation of CMV promoters by these wild-type and mutant proteins revealed common and unique protein domains involved in activation and/or repression of transcription. From the data presented in this study, we propose a model for CMV gene activation by IE gene products.

MATERIALS AND METHODS

Enzymes. Enzymes were obtained from New England BioLabs, Inc. (Beverly, Mass.), Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), Boehringer Mannheim Biochemicals (Indianapolis, Ind.), or Pharmacia LKB Biotechnology (Piscataway, N.J.) and were used as recommended by the supplier or according to standard protocols described elsewhere (37, 38).

Recombinant plasmid DNAs. The plasmid pSVCC3 is derived from pSVCC2 and has been described previously

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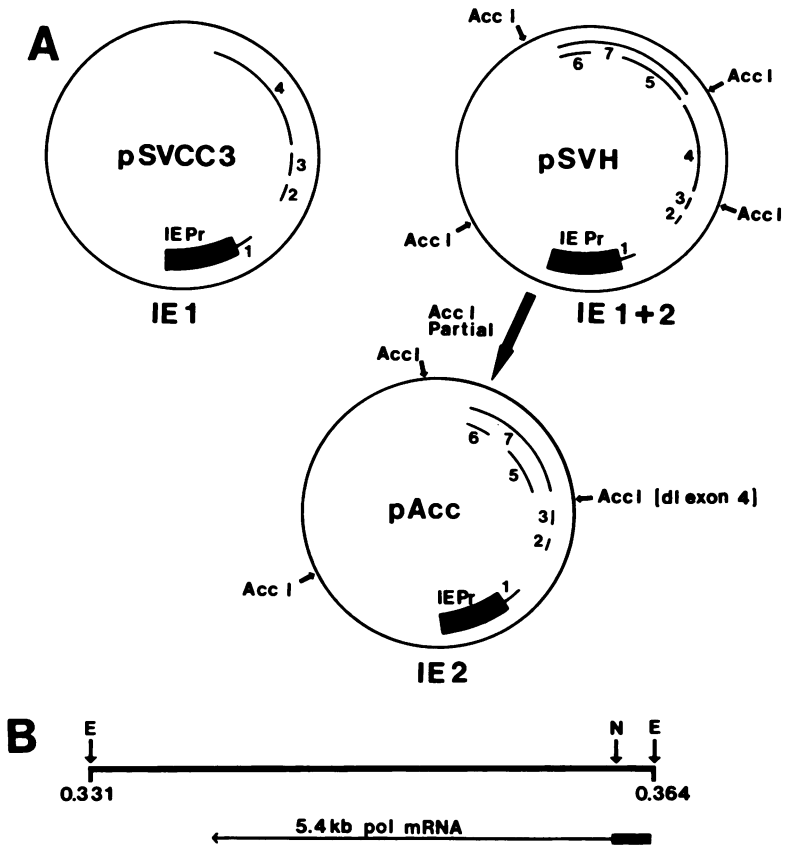

FIG. 1. (A) Structure of the IE1 and IE2 expression vector pSVH. Exons representing IE1 (1, 2, 3, and 4) and IE2 (1, 2, 3, 5, 6, and 7) are indicated and as described previously (38). Sequence is numbered beginning at 40 nucleotides upstream of exon 1 (37). The construction of pSVCC3, pAcc, and pSVH is as described in the Materials and Methods. The IE promoter (IE Pr) is indicated. (B) Structure of the DNA polymerase gene and promoter fragment (15, 21). The polymerase promoter from *Eco*RI (E) to *Nsi*I (N) was cloned into pSVOCAT and used for transfection of HF cells. Map units showing the location of the *pol* gene and the relative location of the *pol* mRNA are indicated at the bottom of the figure. kb, Kilobase.

(9). The plasmid pSVH (Fig. 1) was constructed by inserting region 2 into the 3′ end of pSVCC3 (9). The plasmid pAcc was constructed by partial digestion of pSVH with *Acc*I to delete exon 4 of IE1 (9). The structures of pSVH, pSVCC3, and pAcc are shown in Fig. 1A. pPolCAT (Fig. 1B) was constructed by isolating the *Eco*RI-to-*Nsi*I fragment containing the DNA polymerase promoter from pCMpol2b (15, 20). *Hin*dIII linkers were added, and the fragment was cloned into pSV0CAT. The plasmid pCAT760 was obtained from M. Stinski and has been described previously (39). All plasmids were grown and purified as described previously (43).

Transfection and CAT assays. Transfections with IE plasmids (5 μg) and promoter-*cat* indicator plasmids (5 μg) were done as previously described with human fibroblast (HF) cells passaged 1:2 24 h earlier (9). Cells were harvested at 44 to 48 h after transfection and assayed for chloramphenicol acetyltransferase (CAT) essentially as described previously (9, 14). Assays were linear for 30 min for up to 50% acetylation.

Mutational analysis. Linker insertion mutants were constructed as described in Fig. 2. The plasmid pSVH was partially digested with either restriction enzyme *Fnu*DII or *Hae*III as previously described (36). *Bam*HI linkers (CGCG GATCCGCG) (2 μg) were added to blunt-ended linear molecules of pSVH (1 μg) in 20 μl of ligase buffer (24) for 4 h at 15°C. The reaction mixture was subsequently diluted to 200 μl in 1× ligase buffer and ligated overnight. The products were transformed into *Escherichia coli* HB101 competent cells and screened with restriction endonuclease *Bam*HI. After the linker insertion was mapped relative to the *Bam*HI site (located in the 3′ end of IE1), mutants were tested for their ability to regulate CMV promoters. Mutants that showed phenotypic differences in *trans* activation were redigested with *Bam*HI, recloned to remove any multiple linkers inserted during the initial cloning, and subsequently retested for activity.

Western immunoblot analysis. To examine steady-state levels of IE proteins, transfected HF cells were washed with Tris-buffered saline (TBS) (30 mM Tris [pH 7.4], 150 mM NaCl) and harvested in lysis buffer (50 mM Tris [pH 7.5], 1% sodium dodecyl sulfate). Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose as described previously (35). Blots were reacted with a 1:1,000 dilution of antipeptide antibody (8528) for 1 h at room temperature. Antipeptide antibody 8528 recognizes amino acids 11 to 28 which are common to IE1 and IE2 proteins and has been described previously (35). After extensive washings with TBS, blots were reacted with a 1:1,000 dilution of horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Southern Biotechnologies) and developed with horseradish peroxidase

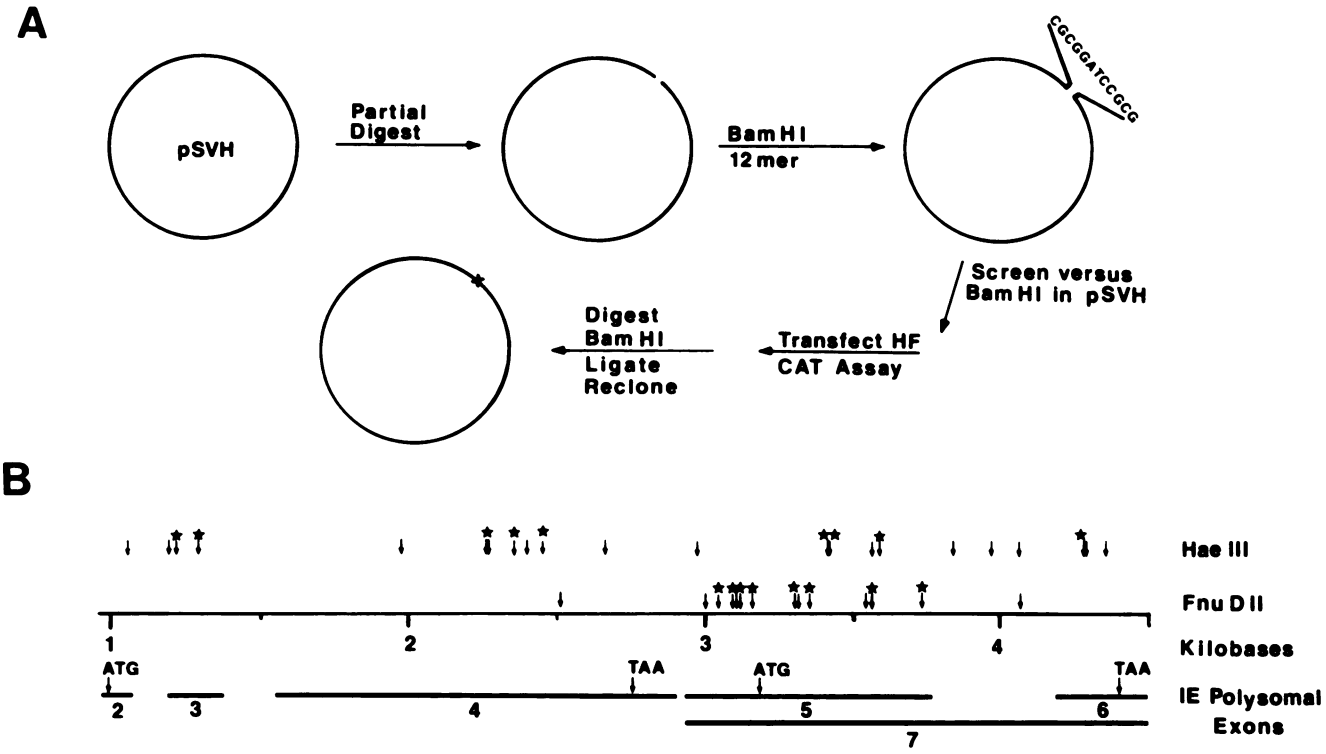

FIG. 2. Linker insertion mutagenesis of IE1 and IE2. (A) pSVH was partially digested with *Fnu*DII or *Hae*III, and linear molecules were isolated and ligated to *Bam*HI linkers (12-mer, CGCGGATCCGCG). Mutants were generated as described in the Materials and Methods. (B) Location of *Fnu*DII and *Hae*III sites (arrows) relative to IE1 and IE2. Insertion mutants obtained and analyzed in this study are indicated with a star.

developing reagent (Bio-Rad Laboratories, Richmond, Calif.) as recommended by the supplier.

Construction of cDNA expression vectors. Synthesis of cDNA to IE mRNAs was accomplished by a variation of the method of Okayama and Berg (30). Briefly, an expression vector was constructed that contains the large *Hin*dIII-to-*Eco*RI fragment of pcDVI and the small *Hin*dIII-to-*Eco*RI fragment of pLI ligated at their appropriate cohesive ends. This vector, designated pSL, contains the simian virus 40 early promoter 5′ to the *Kpn*I site. Vector-primer DNA was prepared exactly as described by Okayama and Berg (30) and used to prime cDNA synthesis. IE cytoplasmic mRNA was prepared as described previously (35, 37, 38). Approximately 2 μg of poly(A)-containing IE mRNA was mixed with 5 μg of vector-primer in 25 μl of 50 mM Tris hydrochloride (pH 8.3)–75 mM KCl–5 mM $MgCl_2$–10 mM dithiothreitol–1 mM each dATP, dCTP, dGTP, and dTTP–500 U of Moloney murine leukemia virus reverse transcriptase. After 1 h at 37°C, the reaction was diluted sevenfold in 25 mM Tris hydrochloride (pH 8.3)–75 mM KCl–5 mM $MgCl_2$–250 μM dATP–250 μM dCTP–250 μM dGTP–250 μM dTTP–5 mM dithiothreitol–250 U of DNA polymerase I per ml–8.5 U of RNase H per ml. The second-strand reaction was incubated at 16°C for 2 h, terminated with 10 mM EDTA, and extracted with phenol-chloroform-isoamyl alcohol (25:25:1). The vector-cDNA was precipitated with ethanol and resuspended, and one-fifth of the product was ligated overnight at 12°C under standard conditions. The ligation was transformed into *E. coli* RR1 and plated on L agar containing ampicillin.

Positive clones were selected by colony blot analysis performed as described previously (24) and hybridized to IE1- or IE2-specific probes previously described (35). Potential IE cDNA clones were subjected to Southern blot analysis and verified by hybridization to IE1 or IE2 probes. cDNAs coding for the IE 72- and 55-kDa proteins were isolated and constructed into a CMV expression vector as described below. These cDNAs were judged "full length" because they contained the *Sac*II site at +71 in exon 1 and consequently were capable of coding for their complete respective proteins. The 86-kDa cDNA was incomplete in that it terminated in exon 2, 18 amino acids short of the amino terminus. The restriction endonuclease pattern of this cDNA was identical to that of the 55-kDa cDNA with respect to sequences 5′ to the IE2 donor splice site which differentiates the 55- and 86-kDa mRNAs (35).

IE1 and IE2 cDNAs were constructed into pSVCC3 and pSVH, respectively, placing the sequences adjacent to their appropriate promoter and 3′ ends. For the IE1 cDNA (pIE72kd), construction employed the *Sac*II site in exon 1 and the *Bam*HI site in exon 4. For pIE55kd cDNA, the *Sac*II site in exon 1 and the *Bsu*36I site in the 3′ end of IE2 were utilized. To generate the pIE86kd expression vector, the sequences between the *Sac*II and *Bsu*36I sites in region 2 were excised from the 86-kDa cDNA and cloned into the analogous sites in pIE55kd. This eliminates the spliced-out region characteristic of the 55-kDa cDNA and effectively constructs the 86-kDa cDNA.

RESULTS

Activation of pPolCAT by IE gene products. To examine the role of IE proteins in activating CMV early gene expression, we cotransfected plasmids capable of expressing IE1

TABLE 1. Activation by IE gene products[a]

Expt	% Acetylation				% of pSVCC3 + pAcc relative to pSVH	Relative increase by pSVCC3 + pAcc over either alone (fold)
	pSVH	pSVCC3	pAcc	pSVCC3 + pAcc		
1	8.9	0.7	0.5	5.6	63	8
2	12.6	1.1	1.1	6.2	49	6
3	8.4	0.7	0.6	4.9	54	7
4	6.7	0.7	0.7	4.3	64	6
Avg	9.1	0.8	0.7	5.3	58	7

[a] HF cells were transfected with 5 μg of pPolCAT and 5 μg of the indicated IE plasmids. In cotransfections with pSVCC3 plus pAcc, 2.5 μg of each plasmid was used. The average of four experiments is indicated.

(pSVCC3), IE2 (pAcc), or both IE1 and IE2 (pSVH) proteins into HF cells with pPolCAT. In addition, IE1 (pSVCC3) and IE2 (pAcc) plasmids were transfected together with pPolCAT in an attempt to reconstruct the effect of pSVH, which expresses IE1 and IE2 proteins. Preliminary studies with pPolCAT demonstrated that the *pol* promoter functions only in the appropriate orientation and that *pol* RNA initiates from the correct 5′ end (data not shown). Also, plasmid vector pSVOd, which does not contain any CMV-specific sequences, is unable to activate pPolCAT to a detectable level (data not shown). The data from four experiments demonstrated that IE1 (pSVCC3)- and IE2 (pAcc)-specific plasmids are unable to activate pPolCAT to significant levels, although some level of expression consistently occurs (Table 1). However, pSVH, which expresses both IE1 and IE2, activated the *pol* promoter to levels significantly higher than IE1 or IE2 alone (approximately 10-fold). Cotransfection of IE1 (pSVCC3) and IE2 (pAcc) also resulted in increased activation of pPolCAT, although at a slightly reduced level when compared with pSVH (approximately six- to eightfold). These data demonstrate that IE1 and IE2 gene products are necessary for complete activation of the early DNA polymerase promoter.

Mutational analysis of IE gene region. To identify the protein domains responsible for IE gene function, we constructed a series of linker insertion mutations within the IE coding region. Figure 2 is a summary of the methodology used to generate the mutants. Mutants were transfected into HF cells in the presence of pPolCAT to compare the relative level of *trans* activation with that of pSVH. Initial analysis of pPolCAT activation by the various mutants demonstrated that regions within both IE1 and IE2 are critical for early promoter activation (Table 2). Within IE2, it appears that the carboxy terminus is critical for early gene expression. Insertion at amino acid 359 (m13) or 540 (m14 and m15) resulted in a significant reduction of activation of pPolCAT (approximately six- to sevenfold). Mutations within the 5′ half of region 2 appeared to have no effect on activation of pPolCAT (amino acids 128 to 310).

Another mutation that affected function was located in exon 3 of IE1 at an *Hae*III site (m2) at nucleotide 1275 (Table 2). This region was common to both IE1 and IE2 and disrupted several IE proteins (72, 86, and 55 kDa) at amino acid 59. This resulted in an 80% inhibition relative to pSVH. In addition, a second mutant (m1), located at amino acid 32, caused a similar reduction in CAT activity. However, this latter mutant was not recloned to remove excess linkers. Also, we tested a number of IE1 insertion (Table 2) and deletion mutants, and all the mutants demonstrated a reduced ability to activate pPolCAT (R. M. Stenberg, unpublished data).

TABLE 2. Effect of IE insertion mutants on promoter function[a]

Target plasmid	Effector plasmid	Mutation (amino acid no.) (IE protein)	% of pSVH	% of pSVOd	% Acetylation
pPolCAT	pSVH				19
	SVHm1	32 (72/86)	23		4
	SVHm2	59 (72/86)	21		4
	SVHm3	128 (86)	84		16
	SVHm4	146 (86)	88		16
	SVHm5	150 (86)	67		13
	SVHm6	154 (86)	81		15
	SVHm7	167 (86)	132		25
	SVHm8	216 (86)	91		17
	SVHm9	252 (86)	90		17
	SVHm10	254 (86)	177		33
	SVHm11	301 (86)	98		18
	SVHm12	310 (86)	112		21
	SVHm13	359 (86)	16		3
	SVHm14	540 (86)	13		3
	SVHm15	540 (86)	11		2
	pSVH				12
	SVHm16	325 (72)	20		2
	SVHm17	325 (72)	28		3
	SVHm18	358 (72)	31		4
	SVHm19	390 (72)	35		4
pCAT760	pSVOd				7
	pSVH			194	13
	pSVCC3			365	25
	pAcc			32	2
	SVHm2	59 (72/86)		96	7
	SVHm3	128 (86)		162	11
	SVHm4	146 (86)		151	10
	SVHm5	150 (86)		135	9
	SVHm6	154 (86)		226	16
	SVHm7	167 (86)		135	9
	SVHm8	252 (86)		184	13
	SVHm11	301 (86)		113	8
	SVHm12	310 (86)		293	20
	SVHm13	359 (86)		1001	69
	SVHm14	540 (86)		1001	69

[a] CAT values are expressed as percent acetylation. All mutants were tested at least twice, and relative values were within experimental error. Numbers presented are from a representative experiment.

Effect of IE1 and IE2 mutants on IE promoter activity. We next analyzed the effect of IE1 and IE2 on IE promoter activity. The plasmid pCAT760 was cotransfected into HF cells with various IE insertion mutants, and the activity of the IE1 promoter was assessed. Table 2 demonstrates that IE1 (pSVCC3) stimulated pCAT760 almost fourfold, while IE2 (pAcc) inhibited activity by 70%. IE1 and IE2 together resulted in a slight net increase (approximately twofold). The various mutants had little effect with the exception of m13 and m14. These mutants failed to downregulate the promoter and resulted in a greatly increased level of IE promoter activation (approximately 10-fold relative to pSVH and 3-fold over that of IE1 alone). The effect of the various IE insertion mutants on activation and repression of CMV promoter function is summarized in Fig. 3.

Analysis of proteins produced by IE mutants. To demonstrate that the effect of the m13 and m14 mutations on activation and repression was not due to an inability to produce IE2 proteins, we analyzed the steady-state levels of

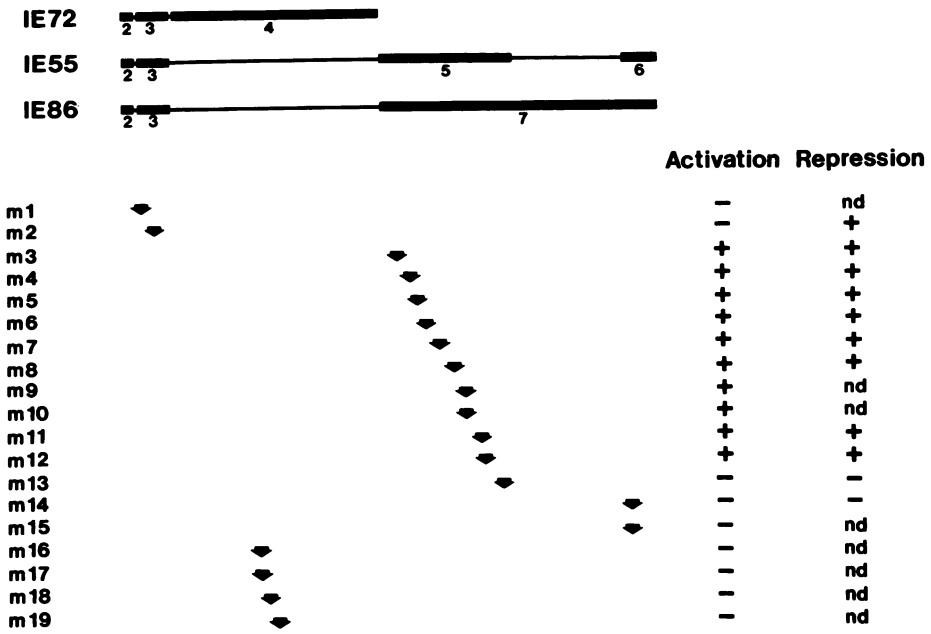

FIG. 3. Activation-repression by IE insertion mutants. The structure of the IE coding regions (thick black lines) and their corresponding intervening sequences (thin black lines) are indicated at the top of the figure. The locations of the various mutants are indicated by the arrows. The ability (+) or inability (−) of each mutant to activate the polymerase promoter or repress the MIEP is indicated. Activation of pPolCAT and repression of pCAT760 are based on values obtained relative to pSVH. nd, Not done.

protein in transfected HF cells. Figure 4 clearly demonstrates that all the IE2 mutants produced normal levels of IE protein relative to pSVH except m13 and m14. These mutants grossly overproduced all IE proteins including the 72-, 86-, and 55-kDa proteins as well as two minor IE1 proteins of 38 and 31 kDa. In contrast, m2 underproduced the 72-kDa protein, which may contribute to its decreased ability to activate the polymerase promoter.

Expression of IE cDNAs in HF cells. To further analyze the role of the IE proteins in regulating viral gene expression, cDNAs capable of expressing individual IE proteins were isolated and cloned into their appropriate IE1 or IE2 vector.

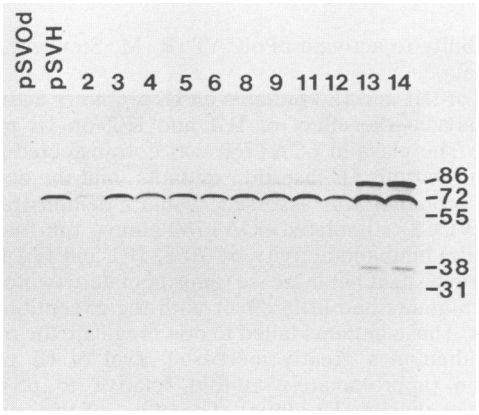

FIG. 4. Proteins expressed by IE insertion mutants. HF cells were transfected with 10 μg of the indicated plasmid, and cells were harvested 48 h later. Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis with antipeptide antibody 8528 (IE1-1). Protein molecular sizes are expressed in kilodaltons on the right. Lane numbers indicate the corresponding mutant.

The structures of the cDNAs and their vectors are shown in Fig. 5A.

IE cDNAs were transfected into HF cells either alone or in combination to test their ability to produce IE proteins. The data in Fig. 5B indicate that pIE55kd and pIE72kd produced significant quantities of their respective proteins. However, pIE86kd produced low to undetectable levels of protein except in the presence of pIE72kd, when it produced protein at significant levels. Also, in the presence of the 86-kDa protein, the level of the 72-kDa protein was reduced when compared with transfection by pIE72kd alone. The relative levels of the 72- and 86-kDa proteins in a mixed transfection approximate the levels seen in a wild-type infection (35).

Activation of pPolCAT by IE proteins. To determine the IE2 proteins responsible for activation of pPolCAT, we transfected the IE2 cDNAs either alone or in the presence of pSVCC3, the IE1 plasmid. Figure 6A demonstrates that IE1 (pSVCC3) and IE2 (pAcc) were necessary for activation of the polymerase promoter. Cotransfection of pSVCC3 with pIE86kd resulted in activation of pPolCAT to levels equivalent to those of pAcc. In contrast, cotransfection of IE1 with pIE55kd did not result in a significant increase in promoter activity. Individually, the IE cDNAs failed to stimulate the polymerase promoter to significant levels.

To extend this study, we cotransfected pSVCC3 with increasing quantities of either pAcc or pIE86kd. The data in Fig. 6B indicate that pIE86kd and pAcc behaved identically with respect to their ability to activate the polymerase promoter.

DISCUSSION

Here we showed that the IE proteins of CMV exhibit promoter-specific differences in their mode of action. The IE proteins have the ability to *trans* activate early promoters while *trans* repressing IE promoters. We identified impor-

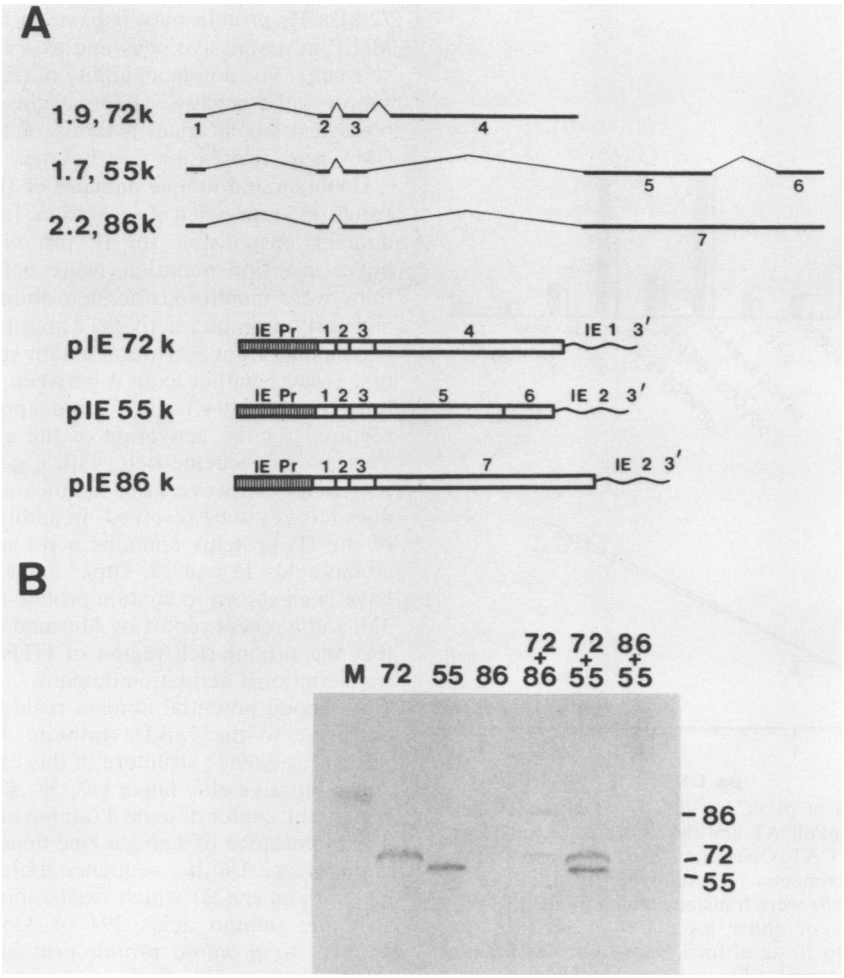

FIG. 5. Structure and expression of IE cDNAs. (A) Structure of IE mRNAs and the corresponding cDNAs. IE cDNAs were cloned 3′ to the IE promoter (IE Pr) and 5′ to the appropriate IE 3′ end. k, Kilodaltons. (B) Proteins expressed by IE cDNAs. HF cells were transfected with a total of 10 μg of the indicated cDNAs, and extracts were prepared at 48 h. Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis with antipeptide antibody 8528 (IE1-1). Protein molecular sizes are expressed in kilodaltons on the right. Lane M, Molecular size markers.

tant regions within these proteins involved in mediating these effects. Furthermore, we cloned cDNA recombinants corresponding to three of the most abundant IE proteins to identify and evaluate critically the structure and function of these IE proteins.

Activation of early promoters. Table 1 demonstrates that the regulation of CMV early gene expression, using the DNA polymerase promoter as a model for activation, requires both IE regions 1 and 2. As previously shown, this is also true for the early-late pp65 phosphoprotein gene, which behaves identically in similar assays (9). Consequently, this may represent a common mechanism for early gene expression during a productive infection. Also, our use of cDNA expression vectors demonstrated that within IE2 the 86-kDa protein is responsible for this activation effect. These results differ from earlier studies with heterologous promoters in either HF (16) or Vero (31) cells which demonstrated that IE2 was sufficient for *trans* activation of promoters. This may be due to the contribution or interaction of cell-specific factors with heterologous or non-CMV promoters. Also, Chang et al. (4) demonstrated that IE2 could activate a CMV early promoter. However, their data also demonstrated that a plasmid containing both IE1 and IE2 activated the CMV early promoter approximately fourfold higher than IE2 alone. This is consistent with our studies (9; this report) which demonstrate low levels of activation of early promoters by IE2 but increased levels of activation by IE1 and IE2. Consequently, we conclude that IE1 and IE2 are synergistic in their activation of CMV early promoters.

Activation and repression of MIEP. Earlier studies demonstrated that the 72-kDa protein was capable of downregulating mRNA levels in nonpermissive COS cells and that an IE1 carboxy-terminal mutant was incapable of this effect (36). Here, we extended those studies and found that IE2 gene products are capable of repressing the activity of the MIEP in permissive HF cells. Analysis of IE protein levels in cells transfected with IE cDNAs indicated that the 86-kDa IE protein is responsible for this effect. In addition, we demonstrated that the IE1 gene product is capable of stimulating the MIEP in HF cells and that IE1 and IE2 proteins together resulted in little net change in expression when compared to the MIEP alone. The finding that IE1 can activate the MIEP is consistent with the recent studies of Cherrington and Mocarski (5). Also, MIEP activity varies in cells under

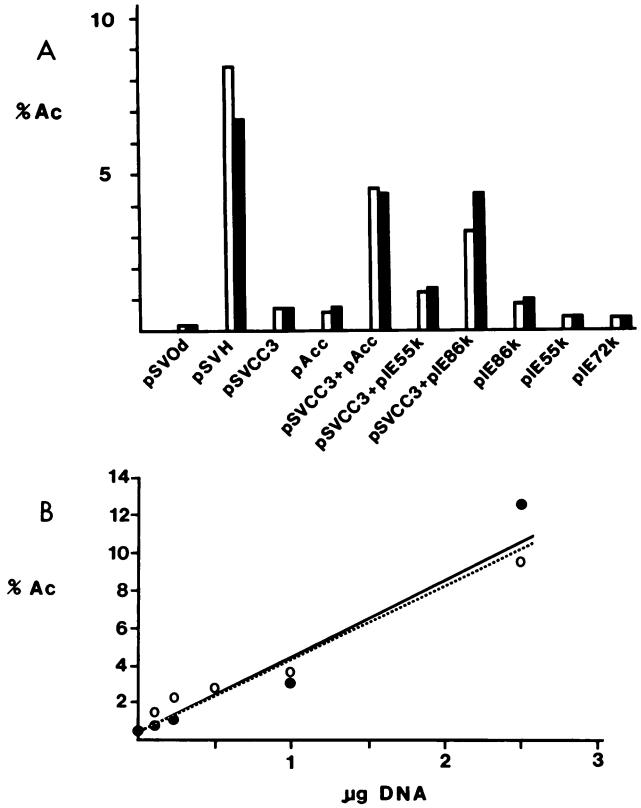

FIG. 6. (A) Activation of pPolCAT with IE cDNAs. HF cells were cotransfected with pPolCAT and the indicated plasmids and subsequently assayed for CAT. Open and closed bars represent the results of duplicate experiments. (B) Activation of pPolCAT by pAcc and pIE86kd. HF cells were transfected with pPolCAT (5 μg) and increasing quantities of either pAcc (○) or pIE86kd (●). Reactions were brought to 10 μg of total DNA by the addition of pSVOd. CAT values are expressed as percent acetylation (% Ac).

permissive and nonpermissive conditions and this effect is due to cell-specific proteins (29). Taken together, these observations may implicate cell-specific components in mediating the function of the IE proteins. In particular, the 72-kDa IE protein may behave as a *trans* activator of the MIEP in permissive cells and as a repressor in nonpermissive cells. The dominant ability of IE1 protein(s) to negate or suppress the negative effects of the IE2 proteins may have biological significance in terms of their ability to regulate CMV gene expression (see below).

Common and unique domains of IE proteins mediate activation and repression of expression. In an effort to identify the domains responsible for IE protein function, a series of linker insertion mutations were constructed. These mutations were monitored for their ability to influence both IE and early promoter activity. Three key regions were identified as important activation and/or repression domains. The first resides within exon 3 between amino acids 32 and 59 (nucleotides 1194 to 1275) and appears to be specifically required for the activation of the early promoter (Fig. 7). This exon is leucine-rich, with a general motif of leucine-X_3-leucine. However, the significance of these leucine residues has yet to be resolved. In addition, the amino terminus of the IE proteins contains a proline-rich region between amino acids 11 and 25. Other nuclear transcription factors have been shown to contain proline-rich regions (1, 2, 6, 22, 33), and a recent report by Mermod et al. (27) demonstrates that the proline-rich region of CTF/NF1 corresponds to a transcriptional activation domain.

A second potential domain resides within exon 4 and is exclusive to the 72-kDa protein. An examination of the primary sequence structure of this exon demonstrates that a single putative zinc finger (17, 28, 32) is located in approximately the center of exon 4 (amino acids 266 to 285) (Fig. 7). The importance of a single zinc finger in the 72-kDa protein is not clear. Another sequence motif present in exon 4 is a leucine zipper (23) which exists adjacent to the zinc finger structure (amino acids 294 to 325) (Fig. 7). This motif appears to promote protein-protein interactions to effect DNA binding. Whether or not this region is capable of forming a leucine zipper structure must await further experimentation. Exon 4 also contains segments of polyglutamic acid, which may form potential acidic activation domains, as well as polyserine stretches, which form potential sites for phosphorylation (13, 19, 47, 48). These homologies may

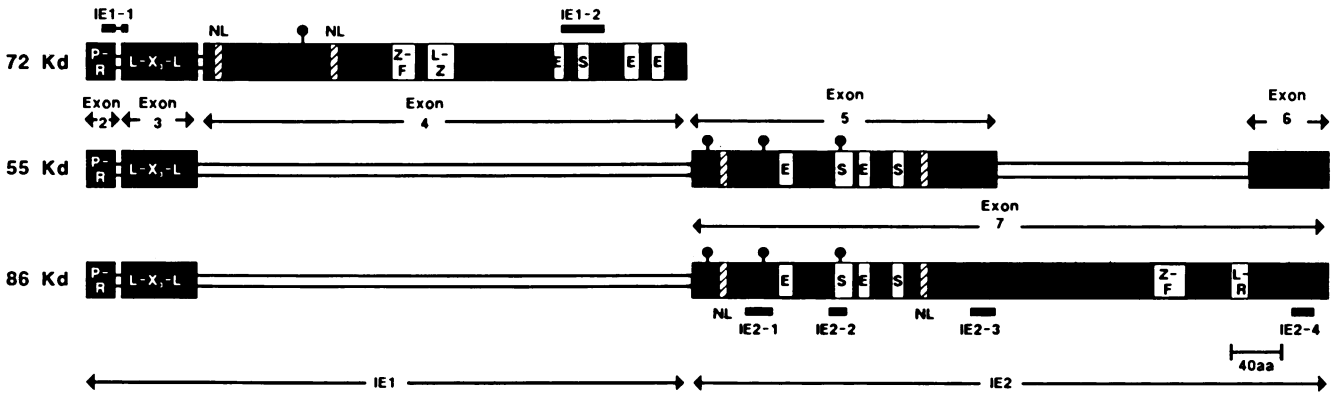

FIG. 7. Linear representation of the structures of the 72-, 55-, and 86-kDa IE proteins. Primary coding structure is represented by the large black boxes, and intergenic regions (not to scale) are shown by the interconnecting bars. The horizontal arrows delineate the exons of the IE1 and IE2 regions. The location of peptides IE1-1 and IE1-2 and IE2-1, -2-2, -2-3, and -2-4, to which antibodies have been prepared (12, 35), are shown by the small black boxes. The scale indicates amino acid (aa) number. Structural features of interest are marked within the coding sequences. Abbreviations: P-R, proline-rich region; L-X_3-L, leucine repeat unit; NL, nuclear localization sequence; Z-F, putative zinc finger motif; L-Z, putative leucine zipper motif; L-R, leucine-rich region; E, polyglumatic acid segment; S, polyserine segment; ♦, potential N-linked glycosylation sites. Kd, Kilodaltons.

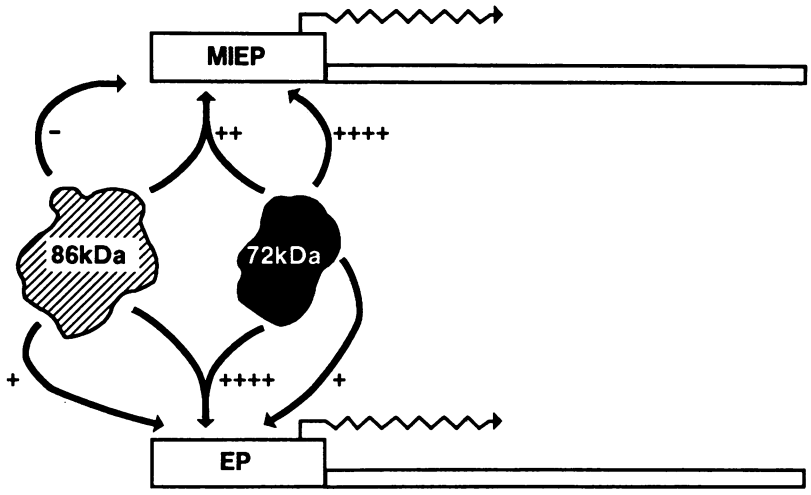

FIG. 8. Model of IE protein function. Influence of the IE 72- and 86-kDa proteins on the MIEP and the early polymerase promoter (EP) is depicted. Direction of transcription from the promoters is indicated by the offset arrows. Arrows from the proteins indicate interaction of the proteins and their subsequent influence on the promoters. The relative levels of activation (+) and repression (−) of the promoters by IE proteins are indicated.

indicate that several distinct units exist within the protein which interact with each other.

The third domain resides within IE region 2 between amino acids 359 and 540 (nucleotides 3721 to 4262). These sequences encompass a putative zinc finger distinct from that present in the 72-kDa protein as well as a leucine-rich domain which weakly conforms to a leucine zipper motif (Fig. 7). This region serves as both an activator of the early promoter and a repressor of the IE promoter. We could not distinguish in the present study whether the activation and repression domains are identical or overlap. However, it does appear that the two mutants that define this domain are phenotypically identical. Clearly, common and unique protein domains within the IE proteins are involved in mediating activation and repression.

Finally, both proteins contain potential nuclear localization signals which are characterized by the clustering of four to six basic amino acids (10) as well as potential N-linked glycosylation sites (25). The locations of the putative regulatory domains relative to the IE proteins are summarized in Fig. 7.

Structure and function of 72-, 86-, and 55-kDa proteins. To determine which IE2 proteins were responsible for activation and/or repression, we isolated cDNA clones corresponding to the predominant IE proteins of 72, 86, and 55 kDa. An analysis of the structure of these clones confirmed our previous observations on the splicing of the IE mRNAs (35, 37, 38). Our studies clearly demonstrated that the 72-kDa protein plays a key role in the activation process. Furthermore, this protein dominantly suppresses the repression effects of the IE2 proteins. The 86-kDa protein is responsible for both the activation and repression effects on the early *pol* promoter and the MIEP, respectively. Mutations in the 86-kDa protein (m13 and m14) affect early promoter activation and IE promoter repression and lead to an increased level of all the IE proteins. The difference in structure between the 55- and 86-kDa IE proteins lies within the IE2 intron, which is an integral component of the 86-kDa protein. This region contains the putative zinc finger and a leucine-rich region (Fig. 7). This strongly associates these regions with the activation-repression by the 86-kDa protein and agrees with the mutagenesis study. These experiments also clearly demonstrated the presence of multiple functional domains within the IE gene region as well as multiple protein involvement in regulating CMV gene expression.

Model for IE protein function. The differential effect of IE proteins on IE and early gene expression in permissive and nonpermissive cells may assist our understanding of the potential mechanisms involved in latency. Negative regulation of the IE promoter by IE1 and IE2 gene products in nonpermissive cells may (i) result in insufficient expression of IE gene products necessary for the maintenance of the replicative cycle or (ii) disrupt the balance of IE gene products, resulting in improper activation of early and late promoters. Our studies demonstrate that the 72- and 86-kDa IE proteins interact to regulate subsequent CMV gene expression (Fig. 8). While the 86-kDa IE protein is involved in both activation and repression, it is clear that its expression is regulated by the presence of the 72-kDa IE protein. This implies that the level of the 72-kDa protein produced in the infected cell is the determining factor in the commitment to activation or repression.

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